Supporting Information

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SI Materials and Methods

Protein Sample Preparation and Mass Spectrometry. To induce senescence, MCF-7 cells were treated with 1 μM doxorubicin for 2 h and then cultured without doxorubicin for 8 d. Senescent and control nonsenescent MCF-7 cells (2×10^8 each) were washed six times with PBS solution, and the medium was changed to DMEM without serum and with non-essential amino acids. The cells were cultured for another 24 h, and the culture supernatant was harvested. The supernatant was centrifuged, filtered through a 0.45-μm filter (Millipore), and concentrated by using a 3,000-Da-cutoff Centriprep spin column (Millipore). The sample was further concentrated by using a 3,000-Da-cutoff Microcon spin column (Amicon). This procedure yielded ~700 μg each protein from senescent and nonsenescent MCF-7 cells, which was used for cleavable isotope-coded affinity tag (ICAT) reagent labeling (senescent cell sample was labeled with isotopically-light ICAT reagent; control nonsenescent cell sample was labeled with isotopically-heavy ICAT reagent). The ICAT-labeled samples were processed and analyzed by microcapillary HPLC/tandem mass spectrometry by using a Thermo Fisher LTQ mass spectrometer as described previously (1–3).

To identify IGFBP3-intercating proteins, forty 15-cm plates of 293T cells were transfected with C-terminally FLAG-His-tagged IGFBP3, and, 24 h later, the culture medium was changed to Opti-MEM (Invitrogen) without serum. Forty-eight hours after transfection, the culture supernatant was harvested, and IGFBP3-FLAG-His and its interacting proteins were purified by nickel agarose chromatography followed by anti-FLAG immunoprecipitation and elution with FLAG peptide. The eluted sample was processed with a Microcon YM-10 column (Millipore) for concentration and buffer change to 50 mM Tris, pH 8.5. The sample was digested with trypsin (Promega) and was analyzed by microcapillary HPLC-tandem mass spectrometry by using a Thermo Fisher Orbitrap mass spectrometer.

Tandem mass spectra were searched against the human IPI protein database using SEQUEST (4). Peptide/protein identification was validated by Peptide/ProteinProphet software tools (5, 6). A ProteinProphet score of 0.9 was used as a cutoff. Protein abundance ratios were calculated by using ASAPRatio software tool (7).

Antibodies. The following antibodies were used for immunoblotting and immunofluorescence: goat polyclonal anti-IGFBP3 (C-19; Santa Cruz Biotechnology), rabbit polyclonal anti-plasminogen activator inhibitor 1 (PAI-1; H-135; Santa Cruz Biotechnology), goat polyclonal anti-PGK1 (Y-12; Santa Cruz Biotechnology), mouse monoclonal anti-Rb (G3-245; BD Pharmingen), rabbit polyclonal anti-p53 (FL-393; Santa Cruz Biotechnology), mouse monoclonal anti-p21 (SX118; BD Pharmingen), mouse monoclonal anti-FLAG (M2; Sigma-Aldrich), rabbit polyclonal anti-FLAG (RFLG-45A; Immunology Consultants Laboratory), mouse monoclonal anti-tubulin (DM1A; Sigma-Aldrich), rabbit monoclonal anti–phospho-histone H3 (3377; Cell Signaling Technology), rabbit polyclonal anti-AKT (9272; Cell Signaling Technology), rabbit polyclonal anti–phospho-AKT-Ser473 (4060; Cell Signaling Technology), rabbit anti-SPARC (H-90; Santa Cruz Biotechnology), mouse monoclonal anti-BrdU (555627; BD Pharmingen), rabbit polyclonal anti-BrdU (600-401-C29S; Rockland Immunochemicals), rabbit polyclonal anti–Ki-67 (15580; Abcam), and rabbit polyclonal anti–trimethylated lysine-9 histone H3 (8898; Abcam).

1. Gygi SP, et al. (1999) Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat Biotechnol* 17:994–999.
2. Shiio Y, et al. (2002) Quantitative proteomic analysis of Myc oncoprotein function. *EMBO J* 21:5088–5096.
3. Lai Y, Qiao M, Song M, Weintraub ST, Shiio Y (2011) Quantitative proteomics identifies the Myb-binding protein p160 as a novel target of the von Hippel-Lindau tumor suppressor. *PLoS ONE* 6:e16975.
4. Eng J, McCormack AL, Yates JR (1994) An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. *J Am Soc Mass Spectrom* 5:976–989.
5. Keller A, Nesvizhskii AI, Kolker E, Aebersold R (2002) Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. *Anal Chem* 74:5383–5392.
6. Nesvizhskii AI, Keller A, Kolker E, Aebersold R (2003) A statistical model for identifying proteins by tandem mass spectrometry. *Anal Chem* 75:4646–4658.
7. Li XJ, Zhang H, Ranish JA, Aebersold R (2003) Automated statistical analysis of protein abundance ratios from data generated by stable-isotope dilution and tandem mass spectrometry. *Anal Chem* 75:6648–6657.

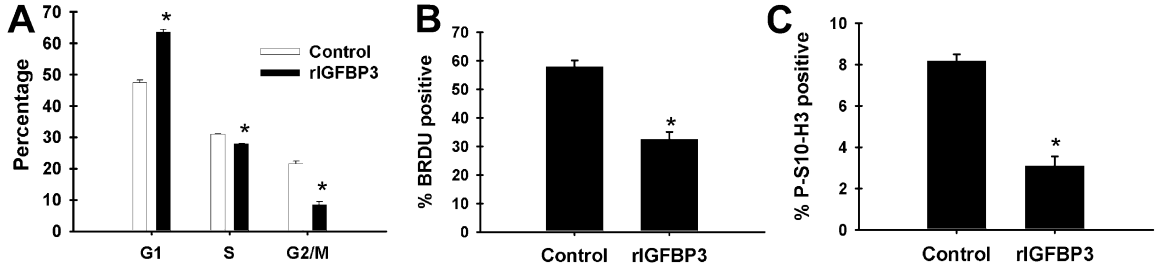

Fig. S1. Effect of recombinant IGFBP3 on MCF-7 cells. (*A*) MCF-7 cells were treated with 0.5 μg/mL recombinant IGFBP3 for 4 d, and the cell cycle profile was determined by FACS analysis (*$P < 0.05$ vs. control.) (*B*) BrdU incorporation (*$P < 0.05$ vs. control). (*C*) Anti–phospho-histone H3 staining (*$P < 0.05$ vs. control).

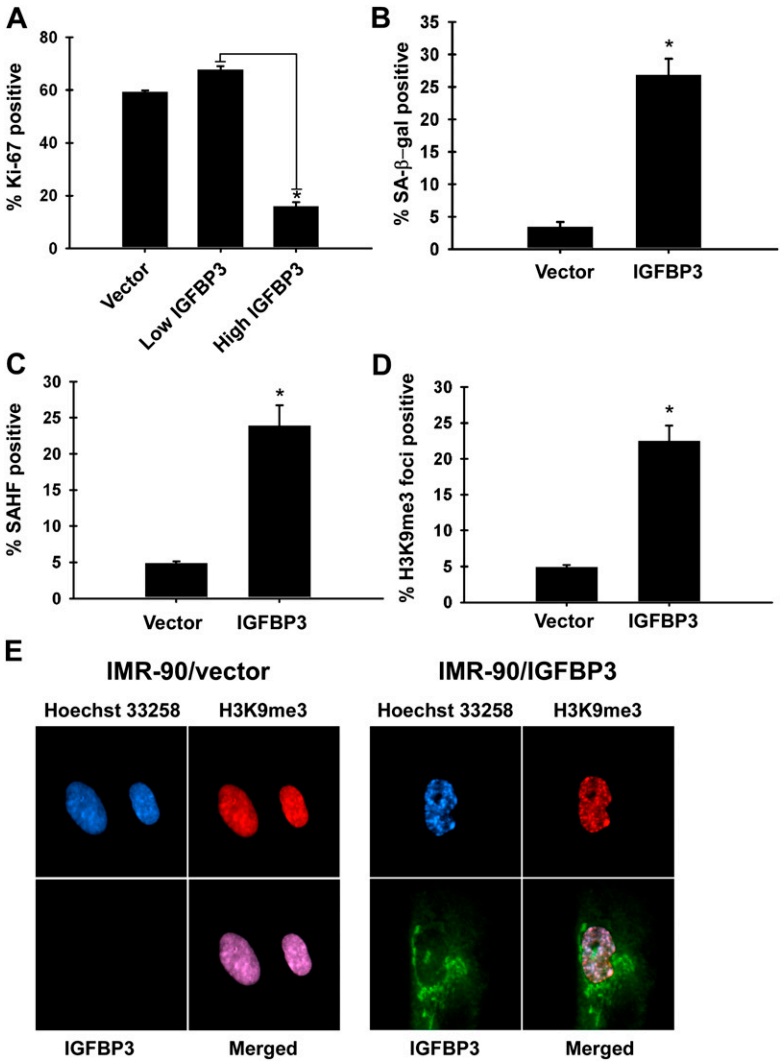

Fig. S2. IGFBP3 induces senescence in IMR-90 fibroblasts. (*A*) IGFBP3 abrogates Ki-67 staining. IMR-90 fibroblasts were infected with IGFBP3-expressing lentivirus or empty vector, and were stained for Ki-67 at 5 d after infection (*$P < 0.05$). (*B*) IGFBP3 induces senescence-associated (SA) β-gal. IMR-90 fibroblasts were infected with IGFBP3-expressing lentivirus or empty vector. Five days after infection, the cells were stained for SA β-gal (*$P < 0.05$). (*C*) IGFBP3 generates senescence-associated heterochromatic foci (SAHF). IMR-90 fibroblasts were infected with IGFBP3-expressing lentivirus or empty vector and the formation of SAHF was assessed by Hoechst 33258 staining (*$P < 0.05$). (*D*) IGFBP3 generates lysine-9 trimethylated histone H3 foci. IMR-90 fibroblasts were infected with IGFBP3-expressing lentivirus or empty vector, and the formation of heterochromatic foci was assessed by anti-trimethylated histone H3 (lysine-9) staining (*$P < 0.05$). (*E*) SAHF formation by IGFBP3. Representative images of the experiments in *C* and *D* are shown.

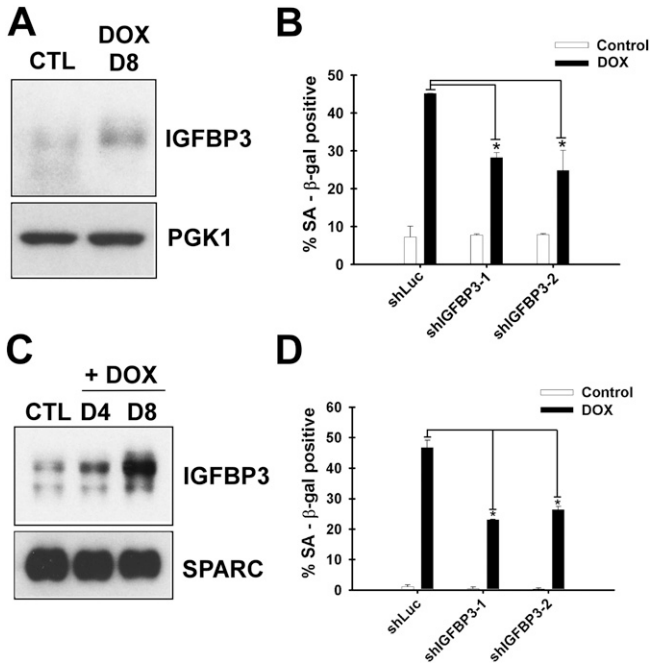

Fig. S3. IGFBP3 mediates doxorubicin-induced senescence in ZR-75-1 and ARPE-19 cells. (*A*) Increased extracellular IGFBP3 upon doxorubicin treatment of ZR-75-1 breast cancer cells. Eight days after doxorubicin treatment of ZR-75-1 cells, conditioned medium was collected and concentrated. Conditioned medium from untreated cells was also included as control. A total of 10 μg of each protein sample was analyzed for IGFBP3 levels by immunoblotting. (*B*) IGFBP3 knockdown alleviates doxorubicin-induced senescence in ZR-75-1 cells. ZR-75-1 cells were infected with lentiviruses expressing shRNA against luciferase or IGFBP3. The cells were treated with 1 μM doxorubicin, and, 8 d later, stained for SA β-gal. (*C*) Increased extracellular IGFBP3 upon doxorubicin treatment of ARPE-19 retinal pigment epithelial cells. Four or 8 d after doxorubicin treatment of ARPE-19 cells, conditioned medium was collected and concentrated. Conditioned medium from untreated cells was also included as control. A total of 10 μg of each protein sample was analyzed for IGFBP3 levels by immunoblotting. (*D*) IGFBP3 knockdown alleviates doxorubicin-induced senescence in ARPE-19 cells. ARPE-19 cells were infected with lentiviruses expressing shRNA against luciferase or IGFBP3. The cells were treated with 1 μM doxorubicin, and, 8 d later, stained for SA β-gal.

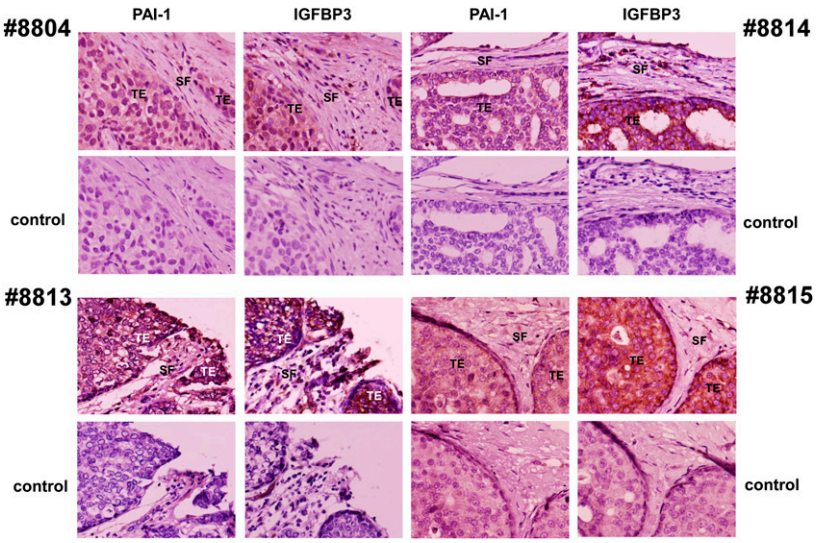

Fig. S4. Immunohistochemical analysis of PAI-1 and IGFBP3 in human breast carcinoma and surrounding tissues. Consecutive paraffin sections of four breast adenocarcinoma samples (cases 8804, 8813, 8814, and 8815) stained for PAI-1 (brown) or IGFBP3 (brown). Control, negative control staining without primary antibody; SF, stromal fibroblasts; TE, tumor epithelial cells.

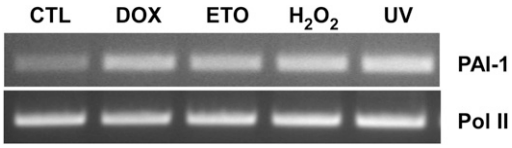

Fig. S5. PAI-1 is induced in response to different senescence-inducing stimuli. MCF-7 cells were treated with 1 μM doxorubicin (DOX) for 2 h, 20 μM etoposide (ETO) for 48 h, 500 μM H_2O_2 for 2 h, or 2 J/m^2 UV light. Four days after initiating each treatment, total RNA was isolated. Total RNA from untreated cells was also included as control (CTL). RT-PCR analysis was performed for mRNA levels of PAI-1 and RNA polymerase II (Pol II; loading control).

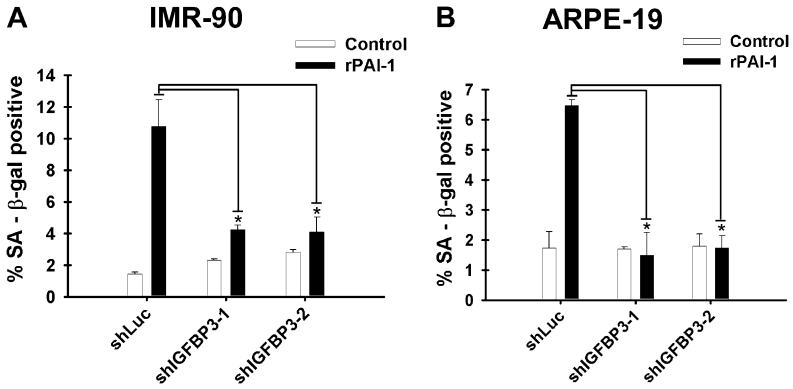

Fig. S6. IGFBP3 mediates PAI-1–induced senescence in IMR-90 and ARPE-19 cells. (*A*) IGFBP3 knockdown abrogates PAI-1–induced senescence in IMR-90 fibroblasts. IMR-90 cells were infected with lentiviruses expressing shRNAs against IGFBP3 or luciferase, treated with recombinant PAI-1 for 4 d, and stained for SA β-gal. (*B*) IGFBP3 knockdown abrogates PAI-1–induced senescence in ARPE-19 retinal pigment epithelial cells. ARPE-19 cells were infected with lentiviruses expressing shRNAs against IGFBP3 or luciferase, treated with recombinant PAI-1 for 4 d, and stained for SA β-gal.

Dataset S1. List of proteins displaying more than twofold increased abundance in senescent MCF-7 conditioned medium

Dataset S1

Protein abundance ratios (senescent MCF-7 conditioned medium/nonsenescent MCF-7 conditioned medium) were calculated using the ASAPRatio software tool. The dataset contains proteins with ProteinProphet probability score ≥0.9.

Dataset S2. List of proteins displaying more than twofold reduced abundance in senescent MCF-7 conditioned medium

Dataset S2

The dataset contains proteins with ProteinProphet probability score ≥0.9.

Dataset S3. List of proteins identified in the IGFBP3-FLAG-His complex

Dataset S3

The dataset contains proteins with ProteinProphet probability score ≥0.9.